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DOCKET NO.: CaCO-0045 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Antoniou and Grosveld

Serial No.: 09/247,054 **Group Art Unit: 1632**

Filing Date: February 9, 1999 **Examiner: Anne-Marie Baker**

For: SELF-REPLICATING EPISOMAL EXPRESSION VECTORS CONFERRING TISSUE-SPECIFIC GENE EXPRESSION

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On July 27, 2001

Doreen Yatko Trujillo

Doreen Yatko Trujillo, Reg. No. 35,719

Assistant Commissioner for Patents
Washington DC 20231

Dear Sir:

COMMUNICATION

The materials I mentioned yesterday are attached and include the following:

1) Information from the ATCC website regarding vectors pXP2 and pT109luc (reporting that they contain the pMB1 origin of replication); and

2) An internet excerpt from *Molecular Genetics of Bacteria*, Larry Snyder and Wendy Champness, ed., ASM Press, Washington, D.C. (indicating that vectors with the pMB1 ori region only replicate in *E. coli* and a few of its close relatives).

Please call when you have had an opportunity to review. Thank you.

Respectfully submitted,

Doreen Yatko Trujillo

Doreen Yatko Trujillo
Registration No. 35,719

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ATCC [redacted]

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pXP2 [redacted]

For query options, please read the search help.

Molecular Biology - Vectors	
ATCC Number:	37577 [redacted]
Designation:	**pXP2**
Depositors:	S.K. Nordeen
Vector:	Polylinker sites: BamHI HindIII SalI SmaI KpnI SstI XhoI BglII Cloning sites: BamHI HindIII SalI SmaI KpnI SstI XhoI BglII Markers: ampR Size (kb): 5.900 Vector type: plasmid Features: enhancer: none promoter: none replicon: pMB1 marker(s): ampR terminator: none insert detection: luciferase
Applications:	shuttle vector promoter-cloning vector One of a series of luciferase vectors (ATCC 37574-37584) for assaying promoter activity in mammalian cells. [RF2160]
Descriptions:	Restriction digests of the clone give the following sizes (kb): BamHI--6.1; HindIII--6.1; SalI--6.1; SmaI--6.1; XhoI--6.1. There is cryptic promoter activity which may be more readily observed with weak promoters. Be careful to do all appropriate controls and map the transcripts. [IV4541] This is a derivative of plasmids patented by the University of California and is not to be used for commercial purposes without prior arrangement with the University of California. [IV4541]
References:	IV4541: Steven K. Nordeen , personal communication RF2160: Nordeen SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques 6: 454-458, 1988 PubMed: 90211866 RF2161: de Wet JR et al. Firefly luciferease gene: Structure and expression in mammalian cells. Mol. Cell. Biol. 7: 725-737, 1987 PubMed: 87144243
Pr pagation:	ATCC medium: 1227 LB Medium (ATCC medium 1065) with 50

	mcg/ml ampicillin Temperature: 37C
BioSafety Level:	1
Shipp d:	freeze-dried Escherichia coli HB101 ATCC 33694
Price:	$145.00
Revised :	Jan 03, 2001

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pT109luc [redacted]

For query options, please read the search help

Molecular Biology - Vectors	
ATCC Number:	37583 [redacted]
Designation:	**pT109luc**
Depositors:	S.K. Nordeen
Vector:	Polylinker sites: BamHI HindIII SalI SmaI KpnI SstI XhoI Cloning sites: BglII Markers: ampR Size (kb): 6.099 Vector type: plasmid Features: enhancer: none replicon: pMB1 marker(s): ampR promoter: HSV TK terminator: none
Applications:	shuttle vector expression vector One of a series of luciferase vectors (ATCC 37574-37584) for assaying promoter activity in mammalian cells. [RF2160]
Descriptions:	Restriction digests of the clone give the following sizes (kb): BamHI--6.6; HindIII--6.6; SalI--6.6. This contains bp -109 to +52 of the herpes simplex tk promoter; both GC + CCAAT are present. [RF2160] There is cryptic promoter activity which may be more readily observed with weak promoters. Be careful to do all appropriate controls and map the transcripts. [IV4541] This is a derivative of plasmids patented by the University of California and is not to be used for commercial purposes without prior arrangement with the University of California. [IV4541]
References:	IV4541: Steven K. Nordeen , personal communication RF2160: Nordeen SK. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques 6: 454-458, 1988 PubMed: 90211866 RF2161: de Wet JR et al. Firefly luciferease gene: Structure and expression in mammalian cells. Mol. Cell. Biol. 7: 725-737, 1987 PubMed: 87144243

Pr pagation:	ATCC medium: 1227 LB Medium (ATCC medium 1065) with 50 mcg/ml ampicillin Temperature: 37C
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Questions or Comments?

Molecular Genetics of Bacteria

Larry Snyder and Wendy Champness

Department of Microbiology
Michigan State University
East Lansing, Michigan

ASM Press
Washington, D.C.

Plasmids

What Is a Plasmid?

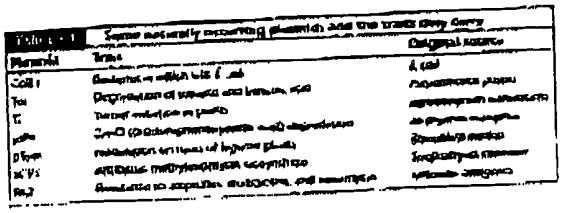
In addition to the chromosome, bacterial cells often contain plasmids. These DNA molecules are found in essentially all types of bacteria and, as we discuss below, play a significant role in bacterial adaptation and evolution. They also serve as important tools in studies of molecular biology. We address such uses later in the chapter.

Plasmids, which vary widely in size from a few thousand to hundreds of thousands of base pairs (a size comparable to that of the bacterial chromosome), are most often circular molecules of double-stranded DNA. However, some bacteria have linear plasmids (see Box 4.1), and plasmids from gram-positive bacteria can accumulate single-stranded DNA owing to aberrant rolling-circle replication (discussed below). The number of copies also varies among plasmids, and bacterial cells can harbor more than one type. Thus, a cell can harbor, for example, two different types of plasmids, with hundreds of copies of one plasmid type and only one copy of the other type.

Like chromosomes, plasmids encode proteins and RNA molecules and replicate as the cell grows, and the replicated copies are usually distributed into each daughter cell when the cell divides. However, unlike chromosomes, plasmids generally do not encode functions essential to bacterial growth. They instead provide gene products that can benefit the bacterium under certain circumstances (see Table 4.1; Box 4.2). In this chapter we'll discuss the molecular basis for these and other features of plasmids.

Naming Plasmids

Before methods for physical detection of plasmids became available, plasmids made their presence known by conferring phenotypes on the cells harboring them. Consequently, many plasmids were named after the genes they carry. For example, R-factor plasmids contain genes for resistance to several antibiotics (hence the name R for resistance). These were the first plasmids discovered, when Shigella and Escherichia coli strains resistant to a number of antibiotics were isolated from the fecal flora of patients in Japan in the late 1950s. The ColE1 plasmid carries a gene for the protein colicin E1, a bacteriocin that kills bacteria that do not carry this same plasmid. The Tol plasmid contains genes for the degradation of toluene, and the Ti plasmid of Agrobacterium tumefaciens carries genes for tumor initiation in plants. This system of nomenclature has led to some confusion, because plasmids carry various genes besides the ones for which they were originally named. Also, we have altered many of these plasmids beyond recognition to make plasmid cloning vectors (see table 4.1) and for other purposes.



To avoid further confusion, the naming of plasmids is now standardized. Plasmids are given number and letter names much like bacterial strains. A small "p." for plasmid, precedes capital letters that describe the plasmid or sometimes give the initials of the person or persons who isolated or constructed it. These letters are often followed by numbers to identify the particular construct. When the plasmid is further altered, a different number is assigned to indicate the change. For example, the plasmid pBR322 was constructed by Bolivar and Rodriguez and is derivative number 322 of the plasmids they constructed. pBR325 is pBR322 with a chloramphenicol resistance gene inserted. The new number 325 distinguishes this plasmid from pBR322.

Functions Encoded by Plasmids

Depending on their size, plasmids can encode a few or hundreds of different proteins. However, as mentioned above, plasmids rarely encode gene products that are always essential for growth, such as RNA polymerase, ribosomal subunits, or enzymes of the tricarboxylic acid cycle. Instead, plasmid genes usually give bacteria a selective advantage under only some conditions.

Table 4.1 lists a few naturally occurring plasmids and some traits they encode, as well as the host in which they were originally found. Gene products encoded by plasmids include enzymes for the utilization of unusual carbon sources such as toluene, resistance to substances such as heavy metals and antibiotics, synthesis of antibiotics, and synthesis of toxins and proteins that allow the successful infection of higher organisms. We can use the fact that plasmids generally carry only nonessential genes to distinguish them from the chromosome, particularly when the plasmid is almost as large as the chromosome.

If plasmid genes, such as those for antibiotic resistance and toxin synthesis, were part of the chromosome, all bacteria of the species, not just the ones with the plasmid, would have the benefits of those genes. Consequently, all the members of that species would be more competitive in environments where these traits were desirable. So why are plasmid genes not simply part of the chromosome? Maybe having some genes on plasmids makes the host species able to survive in more environments without the burden of a larger chromosome. Bacteria must be able to multiply very quickly under some conditions to obtain a selective advantage, and smaller bacterial chromosomes can replicate faster than larger ones. Plasmids encoding

different traits can then be distributed among different members of the population, where they do not burden any single bacterium too heavily. However, if the environment abruptly changes s that the genes carried on one of the plasmids become essential, the bacteria that carry the plasmid will suddenly have a selective advantage and will survive, thereby ensuring survival of the species. In this way, plasmids allow bacteria to occupy a larger variety of ecological niches and contribute to the evolutionary success of not only the bacterial species but also the plasmids found in that species.

Plasmid Structure

Most plasmids are circular with no free ends. All of the nucleotides in each strand are joined to another nucleotide on each side by covalent bonds to form continuous strands that are wrapped around each other. Such DNAs are said to be covalently closed circular (CCC). This structure prevents the strands from separating, and there are no ends to rotate, so that the plasmid can be supercoiled. As discussed in chapter 1, in a DNA that is supercoiled, the two strands are wrapped around each other more or less often than once in about 10.5 base pairs, as predicted from the Watson-Crick double-helical structure of DNA. If they are wrapped around each other more often than once every 10.5 base pairs, the DNA is positively supercoiled; if they are wrapped around each other less often, the DNA is negatively supercoiled. Like the chromosome, covalently closed circular plasmid DNAs are usually negatively supercoiled (see chapter 1). Because DNA is stiff, the negative supercoiling introduces stress, and this stress is partiallN relieved by the plasmid wrapping up on itself, as illustrated in Figure 4 1. In the cell, the DNA wraps around proteins, which relieves some of the stress The remaining stress facilitates some reactions involving the plasmid, such as separation of the two DNA strands for replication or transcription

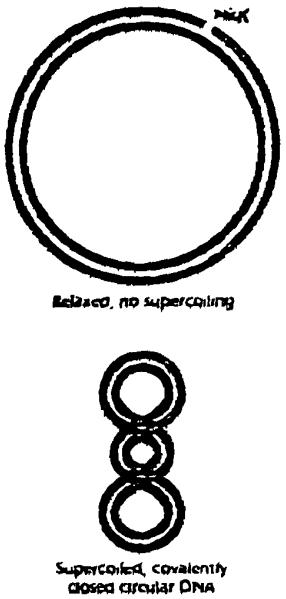


Figure 4.1 Supercoiling of a covalently closed circular plasmid. A break in one strand relaxes the DNA, eliminating the supercoiling.

Properties of Plasmids

Replication

To exist free of the chromosome, plasmids must have the ability to replicate independently DNA molecules that can replicate autonomously in the cell are called replicons. The concept of a replicon was first proposed in a paper by Jacob et al. in 1963 (see Suggested Reading). Plasmids, phage DNA, and the chromosomes are all replicons, at least in the same type of cells.

To be a replicon in a particular type of cell, a DNA molecule must have at least one origin of replication, or ori site, where replication begins (see chapter 1). In addition, the cell must contain the proteins that enable replication to initiate at this site. Plasmids encode only a few of the proteins required for their own replication. In fact, many encode only one of the proteins needed for initiation at the ori site. All of the other required proteins, including DNA polymerases, ligases, primases, helicases, and so on, are borrowed from the host.

Each type of plasmid replicates by one of two general mechanisms (Figure 4.2), which is determined along with other properties* by the genes surrounding the molecule's ori (see the section on the ori region, below). The plasmid replication origin is named oriV (ori vector). Most of the evidence for the mechanisms described below came from observations of replicating plasmid DNA with the electron microscope.

THETA REPLICATION

Some plasmids begin replication by opening the two strands of DNA at the ori region, creating a structure that looks like the Greek letter θ—hence the name theta replication (Figure 4.2). In this process, an RNA primer begins replication, which can proceed in one or both directions around the plasmid. In the first case, a single replication fork moves around the molecule until it returns to the origin and then the two daughter DNAs separate. In the other case (bidirectional replication), two replication forks move out from the ori region, one in either direction, and replication is complete (and the two daughter DNAs separate) when the two forks meet somewhere on the other side of the molecule.

The theta mechanism is the most common form of DNA replication. It is used not only by most plasmids,

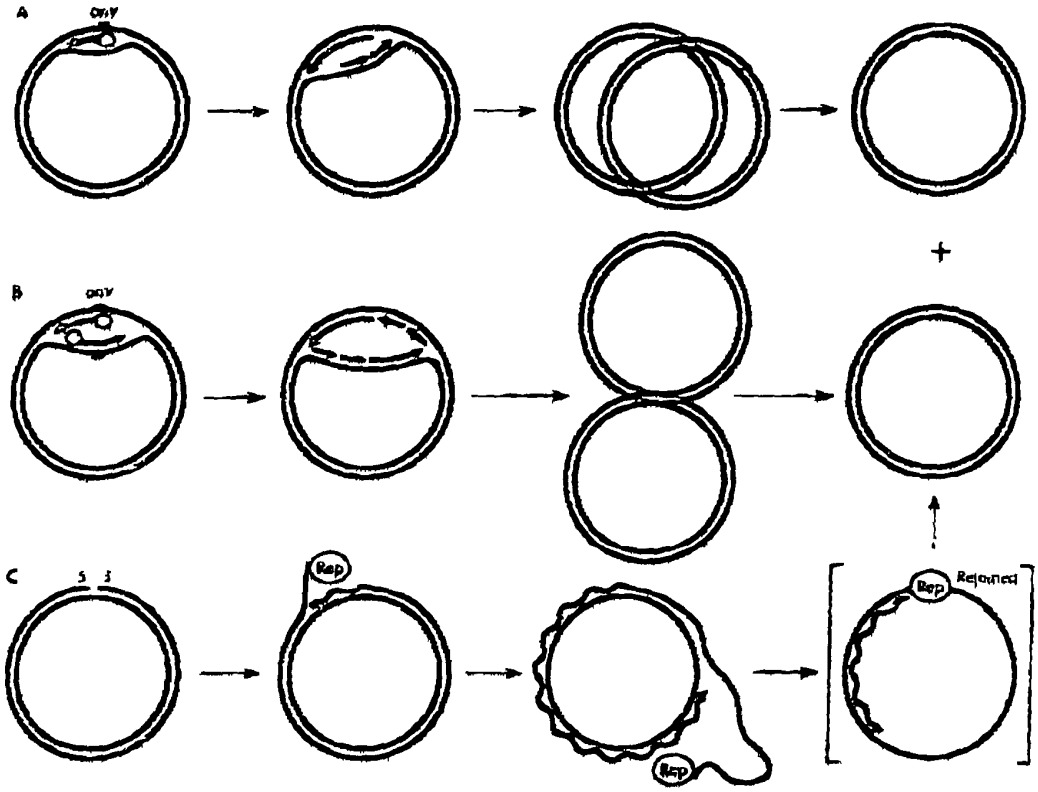


Figure 4.2 Some common schemes of plasmid replication. The origin region is designated *ori*. (A) Unidirectional replication. Replication terminates when the replication fork gets back to the origin. (B) Bidirectional replication. Replication terminates when the replication forks meet somewhere on the DNA molecule opposite the origin. (C) Rolling-circle replication. Replication from the 3 end at a nick displaces one of the two strands. The ends of the displaced strand are rejoined, and the complement of the displaced strand is made from RNA primers synthesized at unique sites.

including ColE1, RK2, F, and P1, but also by the chromosome (see chapter 1).

ROLLING-CIRCLE REPLICATION

In the other type of replication, one strand is nicked at the ori region and the 3' OH end at the nick serves as a primer for replication. The displaced strand can then serve as a template to make another double-stranded DNA molecule. The term rolling-circle replication describes how the template strand can be imagined to roll as it replicates (Figure 4.2)

To begin, rolling-circle replication requires a nick in one strand of the double-stranded replicative form (RF) at the so-called plus origin. Then replication proceeds around the circle, displacing the opposite, or minus, strand. On the displaced strand, replication initiates at specific sites, "minus-strand origins," to make another double-stranded replicative form. If these minus-strand origins do not function well in a particular host, single minus strands will accumulate. In fact, the so-called singled-stranded plasmids found in some gram-positive bacteria have large amounts of single-stranded DNA, apparently as a result of defective rolling-circle replication.

Plasmids that replicate by a rolling-circle mechanism include pUB110 and pC194, isolated from Staphylococcus aureus, and pIJ101, isolated from Streptomyces lividans. Some types of phage also replicate by this mechanism (see chapter 7).

Functions of the ori Region

In most plasmids, the genes for proteins required for replication are located very close to the ori . sequences at which they act. Thus, only a very small region surrounding the plasmid ori site is required for replication. As a consequence, the plasmid will still replicate if most of its DNA is removed, provided the ori region remains and the plasmid DNA is still circular. Smaller plasmids are easier to use as cloning vectors, as discussed later in the chapter.

In addition, the genes in the ori region often determine many other properties of the plasmid. Therefore, any DNA molecule with the ori region of a particular plasmid will have other characteristics of that plasmid. The following sections describe the major plasmid properties determined by the ori region.

HOST RANGE

The host range of a plasmid includes all the types of bacteria in which the plasmid can replicate, and the host range is usually determined by the ori region. Some plasmids, such as those with ori regions of the ColE1 plasmid type, including pBR322 and pET and pUC, have narrow host ranges. These plasmids will replicate only in E. coli and some other closely related bacteria such as Salmonella and Klebsiella species. In contrast, plasmids with a broad host range include the RK2 and RSF1010 plasmids, as well as the rolling-circle plasmids from gram-positive bacteria mentioned above. The host range of these plasmids is truly remarkable. Plasmids with the ori region of RK2 will replicate in most types of gram-negative bacteria, and RSF1010-derived plasmids will even replicate in some tyes of gram-positive bacteria. Many of the plasmids isolated from gram-positive bacteria also have quite broad host ranges. For example, pUB110, which was first isolated from the gram-positive S. aureus, will replicate in many other gram-positive bacteria, including Bacillus subtilis. However, most plasmids isolated from gram-negative bacteria will not replicate in gram-positive bacteria and vice versa, which reflects the evolutionary divergence of these groups (see the introductory chapter).

It is perhaps surprising that the same plasmid can replicate in bacteria which are so distantly related to each other. Broad-host-range plasmids must encode all of their own proteins required for initiation of replication, and so they do not have to depend on the host cell for any of these functions. They also must be able to express these genes in many types of bacteria. Apparently, the promoters and ribosome initiation sites for the replication genes of broad-host-range plasmids have evolved so that they will be recognized in a wide variety of bacteria.

REGULATION OF COPY NUMBER

Another characteristic of plasmids that is determined mostly by their ori region is their copy number, or the average number of a particular plasmid per cell. More precisely, we can define the copy number as the number of copies of the plasmid in a newborn cell immediately after cell division. All plasmids must regulate their replication; otherwise they would fill up the cell and become too great a burden for the host, or their replication would not keep up with the cell replication and they would be progressively lost during cell division. Some plasmids, such as pIJ101 of Streptomyces coelicolor, replicate enough to populate the cell with hundreds of copies. However, others, such as the F plasmid of E. coli, replicate only once or a few times during the cell cycle. Table 4.2 lists the copy numbers of these and other plasmids.

TABLE 4.2 Copy numbers of some plasmids

Plasmid	Approximate copy number
F	1
P1 prophage	1
RK2	4–7 (in *E. coli*)
pBR322	16
pUC18	~30–50
pIJ101	40–300

The regulation mechanisms used by plasmids with higher copy numbers often differ greatly from that used by plasmids with lower copy numbers. Plasmids that have high copy numbers, such as ColE1 plasmids, need only have a mechanism that inhibits the initiation of plasmid replication when the number of plasmids in the cell reaches a certain level. Consequently, these molecules are called relaxed plasmids. By contrast, low-copy-number plasmids such as F must replicate only once or very few times during each cell cycle and so must have a tighter mechanism for regulating their replication. Hence, these are called stringent plasmids. Much more is understood about the regulation of replication of relaxed plasmids than about the regulation of replication of stringent plasmids. The following paragraphs describe some of the better-known examples.

Mechanisms To Prevent Curing of Plasmids

Cells that have lost a plasmid during cell division are said to be cured of the plasmid. Several mechanisms prevent curing, including plasmid addiction systems (see Box 4.3, Plasmid Addiction), site-specific recombinases that destroy multimers, and partitioning systems.

RESOLUTION OF MULTIMERIC PLASMIDS

The possibility of losing a plasmid during cell division is increased if the plasmids form multimers during replication. A multimer consists of individual copies of the plasmid molecules linked to each other. The multimers probably occur as a result of defects in replication termination or by recombination of monomers. The formation of multimers effectively lowers the effective copy number because each multimer will segregate into the daughter cells as a single plasmid. Therefore, multimers greatly increase the chance of a plasmid being lost during cell division.

To avoid this problem, many plasmids have site-specific recombination systems that destroy multimers. These systems promote recombination between specific sites on the plasmid if the same site occurs more than once in the molecule, as it would in a multimer. This recombination has the effect of resolving multimers into separate molecules.

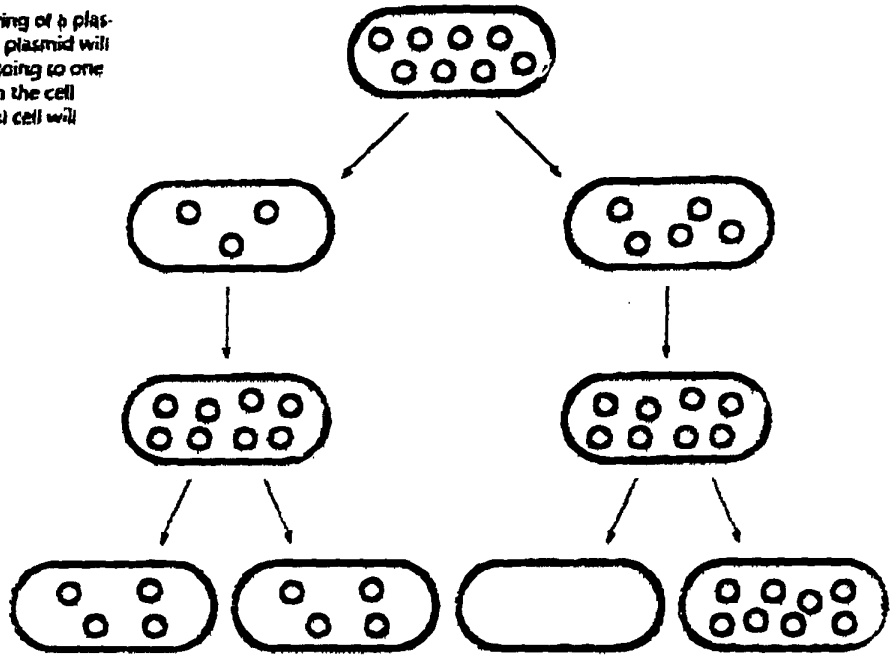


Figure 4.8 Random curing of a plasmid with no *par* site. Each plasmid will have an equal chance of going to one of the daughter cells when the cell divides, and the occasional cell will inherit no plasmids.

The best known example of this mechanism is the cer-Xer site-specific recombination system of the ColE1 plasmid (see Guhathakurta et al., Suggested Reading). The cer site is analogous to the dif site (discussed in chapter 1), and the same Xer proteins act on this site as act on the dif site to resolve the bacterial chromosomes after replication and allow them to separate into the daughter cells. The Xer proteins are site-specific recombinases because they act only on specific sites, the dif site in the chromosome and the cer site in ColE1 plasmids. We discuss site-specific recombinases in more detail in chapter 8.

PARTITIONING

Plasmids also avoid being lost from dividing cells by carrying **partitioning** systems, which ensure at least one copy of the plasmid segregates into each daughter cell during cell division. The functions involved in these systems are called *par* functions.

Using combinatorial probability, we can calculate how often cells will be cured of a plasmid if the plasmid has no par system. In the simple example shown in Figure 4.8, the copy number of the plasmid is 4. Immediately after cell division, a cell contains four copies, and immediately before the cell divides, it contains eight copies. If the plasmids are equally divided into the two daughter cells, each will get four plasmids. However, the plasmids usually will not be equally distributed between the two daughter cells, and one daughter cell will get more than the other. In fact, with a certain probability, one cell will get all of the plasmids and the other cell will be cured. Since each plasmid can go into either one cell or the other, the probability that one daughter cell will be cured of the plasmid is the same as the probability of tossing

eight heads (or tails) of a coin in a row. Thus, the probability that the first plasmid will go into one cell is 1/2, and the probability that the first two plasmids will go into the same cell is 1/2 times 1/2 = 1/4, and so on. The probability that all eight will go into one cell is therefore $(1/2)^8$ or 1/256. Since it is irrelevant which of the two cells is cured, the frequency of curing will be twice this value, so $2(1/2)^8$ or 1/128 of the cells will be cured each time the cells divide. In general, for a plasmid with a copy number n, the frequency of curing is $2(1/2)^{2n}$, since the number of plasmids at the time of division is twice the copy number. Also, as the cells divide once every generation time, the frequency of cured cells in the population will be roughly equal to the number of generation times that have elapsed times this number. This is the frequency of curing if the sorting of the plasmids into daughter cells is completely random. Therefore, if the fraction of the cells that are cured of the plasmid is less than $2(1/2)^{2n}$ times the number of generation times that have elapsed, the plasmid must have some sort of partitioning function.

This calculation indicates that few cells would be cured of a high-copy-number plasmid each generation, even without a partitioning mechanism. However, a significant fraction of cells would be cured of a low-copy-number plasmid each generation. In fact, with a plasmid with a copy number of only 1, such as F or P1, $2(1/2)^2$ or 1/2 of the cells would be cured each generation. Since cells are

seldom cured of even low-copy-number plasmids, some mechanism must ensure that plasmids, especially those with low copy numbers, will be partitioned faithfully into the daughter cells each time the cell divides

The *par* Functions

Some plasmids, including F, P1, and R1, have short regions that are known to enhance proper partitioning. If such a region is removed from the plasmid, the frequency of curing will be much higher, close to that predicted above. The partitioning system of the P1 plasmid has been studied most extensively. The P1 plasmid par region consists of a cis-acting par sequence and the genes for two proteins, ParA and ParB, one of which binds to the par site.

Mechanism of Partitioning

Figure 4 9 shows two general ideas for how par sites might ensure proper partitioning of a plasmid (see Austin and Nordstrom, Suggested Reading). According to these models, the bacterial membrane functions like the mitotic spindle in eukaryotic organisms, pulling plasmids apart prior to cell division According to both models, the par site is the region of the plasmid that binds to the cellular membrane, and as the membrane grows during cell division, the two copies of the plasmid are pulled apart into the two daughter cells. The two models differ in explaining how binding to the membrane ensures that at least one copy of the plasmid enters each daughter cell at the time of division.

According to the model shown in Figure 4.9A, the par sites bind to putative sites on the bacterial membrane. In the model, each type of plasmid has its own unique site on the membrane. As the cell grows, these sites will duplicate and bind only one copy of the plasmid. Because these sites on the membrane move apart as the cell grows and divides, one copy of the plasmid will be distributed into each of the daughter cells. Even if there are more copies of the plasmid and these are randomly distributed during division, the cell will not be cured provided that at least one copy of the plasmid is bound to the membrane of each daughter cell.That single copy can then multiply many times before the next division.

The problem with this model is the required number of unique sites on the membrane--one for each type of plasmid. It seems unlikely that there would be as many unique sites on the membrane as there are different types of plasmids with partitioning sites.

Model A remains tenable yet avoids the requirement for unique membrane sites if we propose that the sites to which the plasmids bind are on the chromosome rather than the membrane. Because of the complexity of sequence in the chromosome, an almost infinite number of unique sites is possible, and these sites will double each time the chromosome replicates. The only unique site on the membrane need be the site to which the

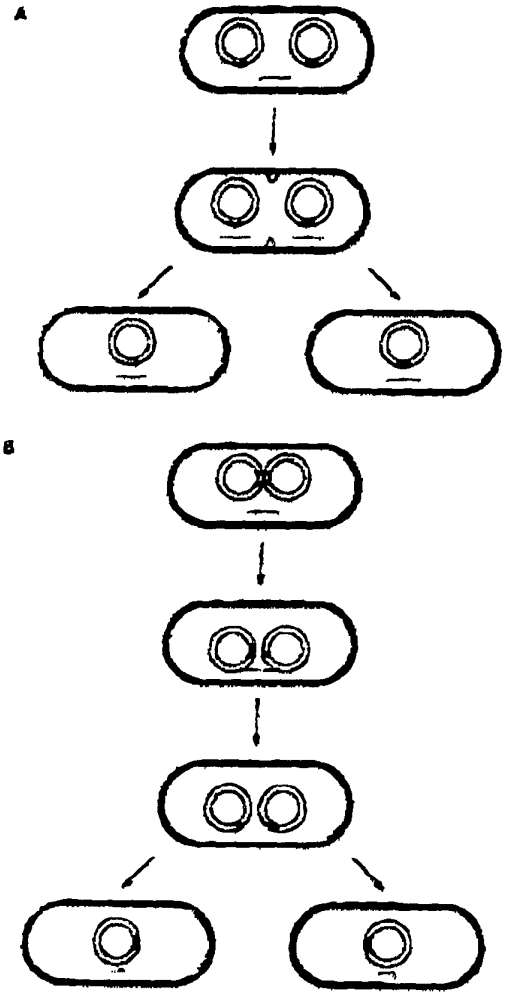


Figure 4.9 Two models for the function of par sites. In model A, the plasmid binds to a unique site on the bacterial membrane and the two copies of the plasmid are pulled apart as the site on the membrane divides. In model B, the two copies of the plasmid bind to each other before they bind to a site on the membrane. One then associates with each site when the site on the membrane divides.

chromosome binds, and the plasmid will partition along with the chromosome.

Model B is similar but avoids the unique-site problem altogether. In this model, two copies of the plasmid must pair with each other at their par sites before the compound par site can bind to the membrane Then the two plasmids separate as the site on the membrane is pulled apart when the cell divides. One copy of the plasmid will partition properly into each of the daughter cells provided that two plasmids will not pair with each other unless they have the same par site and that only paired par sites will bind to the membrane.

High-copy-number plasmids often have sites that increase proper partitioning of the plasmid, but these regions do not have the same structure and may not work by the same mechanism. For example, the par region of pSC101 increases supercoiling of this plasmid. How increased supercoiling aids in proper partitioning is not clear but perhaps it enhances replication of plasmids immediately after division so that they are less apt to be cured in the next division.

Incompatibility

Many bacteria, as they are isolated from nature, contain more than one type of plasmid. These plasmid types stably coexist in the bacterial cell and remain there even after many cell generations. In fact, bacterial cells containing multiple types of plasmids will not be cured of each plasmid any more frequently than if the other plasmids were not there.

However, not all types of plasmids can stably coexist in the cells of a bacterial culture. Some types will interfere with each other's replication or partitioning so that if two such plasmids are introduced into the same cell, one or the other will be lost at a higher than normal rate when the cell divides. This phenomenon is called plasmid incompatibility, and two plasmids that cannot stably coexist are members of the same incompatibility, or Inc, group. If two plasmids can stably coexist, they belong to different Inc groups. There may be hundreds of different Inc groups, and plasmids are usually classified by their group. For example, RP4 and RK2 are both IncP (incompatibility group P) plasmids. In contrast, RSF1010 is an IncQ plasmid, so can be stably maintained with either RN or RK2 because it belongs to a different Inc group.

Plasmids of the same Inc group can be incompatible because they share the same replication control mechanism and/or because they share the same partitioning (*par*) functions.

Plasmid Genetics

Methods for Detecting and Isolating Plasmids

The many methods developed for detecting plasmids generally take advantage of the small size and/or the circularity of these molecules.

ALKALI LYSIS METHOD

The alkali lysis method takes advantage of the relatively small size of plasmids to separate them from chromosomal DNA. First, treatment of the cell lysate with alkali results in separation, or denaturation, of the DNA strands. Then increasing the salt concentration causes the chromosomal DNA, which is very large and bound to proteins, to precipitate.

The relatively small plasmids do not precipitate readily at high salt concentrations and remain in the supernatant. Because plasmids are covalently closed, their DNA strands cannot completely separate if the DNA is denatured. Their intertwining also allows the two plasmid DNA strands to quickly find their complementary sequences and renature to form double-stranded DNA once the denaturing conditions are removed. The chromosome, in contrast, will not quickly renature. While circular, the chromosome is so large that it is usually broken at early stages of purification procedures, so that the strands separate in the presence of a denaturant. Also, because the chromosome is so large, the complementary sequences take hours to renature once they have been denatured.

CsCl-EtBr PURIFICATION

Other methods for purifying plasmids are based on the fact that covalently closed circular chromsomes bind less ethidium bromide (EtBr) than do linear or nicked circular DNAs (Figure 4.11). EtBr intercalates (inserts itself) between DNA bases, pushing the bases apart and rotating the two strands of the DNA around each other. The two strands of the covalently closed circular plasmid are not free to rotate, and the binding of EtBr therefore increases the stress on the DNA until no more EtBr can bind. In contrast, because the strands of the chromosomal DNA have usually been broken during the first steps of the purification procedure, they are free to rotate and chromosomal DNA can bind much higher concentrations of EtBr.

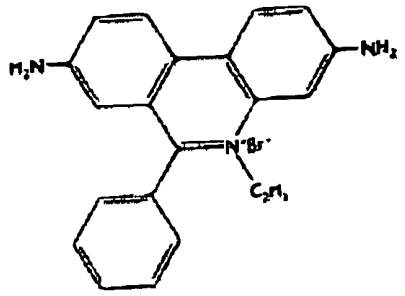
EtBr bound to DNA makes it less dense in salt solutions made with heavy atoms such as cesium chloride (CsCl). As a consequence, if the DNA is mixed with a solution of CsCl and EtBr and centrifuged to establish a gradient of CsCl concentration, the covalently closed circular plasmid DNAs will band lower, at a position where the solution is more dense (see Figure 4.12).

ELECTROPHORESIS

The methods discussed above work well with plasmids that have many copies per cell and are not too large.

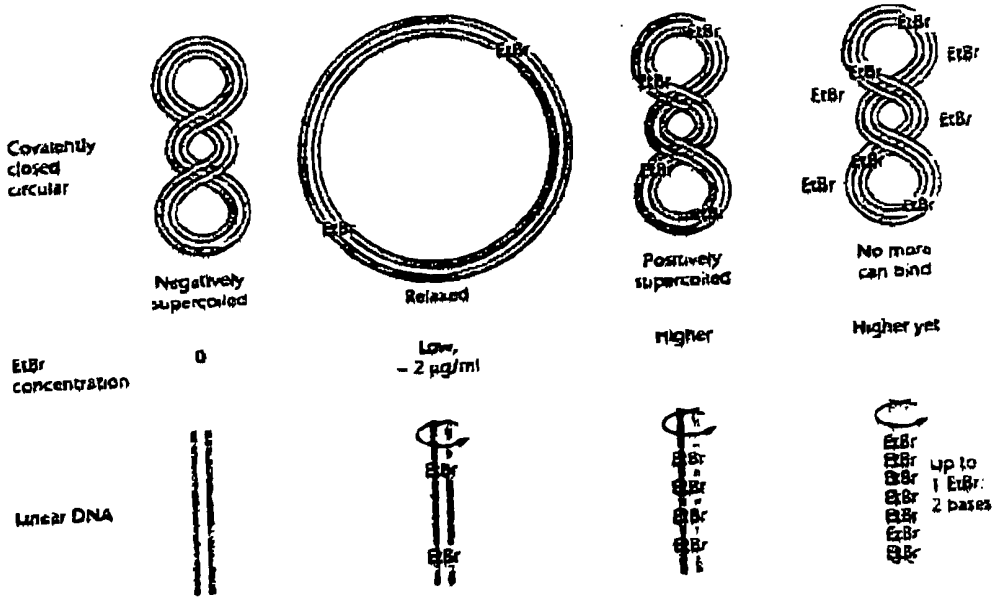
However, large, low-copy-number plasmids are much more difficult to detect, and other methods must be used. Large plasmids are much more susceptible to breakage than are smaller plasmids. Consequently, the cell-lysing procedures must be gentle. Also, like chromosomes, large plasmids will precipitate at high salt concentrations and will not renature spontaneously after the strands have separated.

Most methods for detecting large plastruds involve separating them from the chromosome directly by electrophoresis on agarose gels. In the example shown in Figure 4.13, the cells were broken open directly on the agarose gel to avoid breaking the plasmid DNA. Then application of an electric field caused the DNA to



Chemical structure of ethidium bromide

Figure 4.11 Less EtBr can bind to a covalently closed circular DNA than t a linear or nicked circular DNA. Also, progressively higher EtBr concentrations shift DNA supercoiling from negative to positive. At about 2 μg of EtBr per mL most DNAs are completely relaxed. The arrow indicates the free rotation of linear DNA.



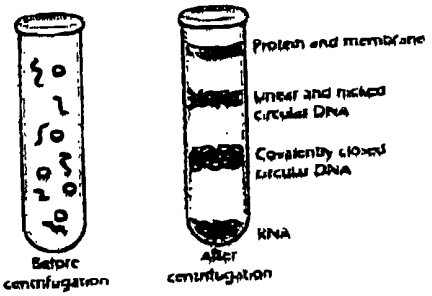


Figure 4.12 Separation of covalently closed circular plasmid DNA from linear and nicked circular DNAs on EtBr-CsCl gradients. After centrifugation, the plasmid DNA will band below the other DNAs because it has a higher buoyant density as a result of less binding of EtBr.

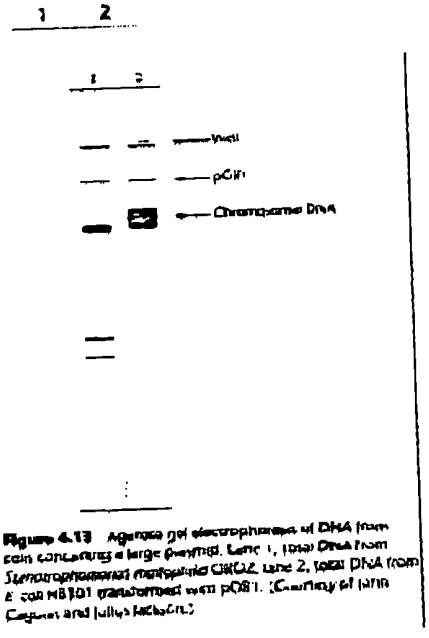


Figure 4.13 Agarose gel electrophoresis of DNA from cells containing a large plasmid. Lane 1, total DNA from *Stenotrophomonas maltophilia* CIRCA2. Lane 2, total DNA from *E. coli* HB101 transformed with pQR1. (Courtesy of John Cleaver and Julie McEwen.)

migrate, or run, along the gel. After electrophoresis, the DNA bands were stained with EtBr, and UV light was shone on the gel to reveal the position of the DNA. EtBr

will fluoresce if it absorbs UV light when bound to DNA. The plasmid, because of its unique size, makes a band on the gel that is distinct from that due to chromosomal DNA.

PULSED-FIELD GEL ELECTROPHORESIS

Standard agarose gels cannot be used to separate larger DNAs. Note that in Figure 4.13 the chromosomal DNAs form one band, even though these molecules have been broken into many pieces of different lengths. Also, although the plasmid is smaller, it runs more slowly (travels a shorter distance) than the chromosome on the gel. The reason for this anomalous behavior is that as long, linear molecules move through the gel, they tend to orient themselves in the direction they are going. They will then move faster and all at the same rate independent of their length. If a piece of DNA is shorter, or circular like the plasmid, it will not orient itself as readily and will travel a shorter distance.

Methods such as pulsed-field gel electrophoresis (PFGE) have been devised to allow the separation of long pieces of DNA based on size These methods depend on periodic changes in the direction- of the electric

field. The molecules will attempt to reorient themselves each time the field shifts, and the longer molecules will reorient themselves more slowly than the shorter ones and so will move more slowly Such methods have allowed the separation of DNA molecules hundreds of thousands of base pairs long and the detection of very large plasmids. We discuss them in more detail in chapter 16.

Plasmid Cloning Vectors

A cloning vector is an autonomously replicating DNA into which other DNAs can be inserted. Any DNA inserted into the cloning vector will then replicate passively with the vector, so that many copies of the original piece of DNA can be obtained. Cloning vectors can be made from essentially any DNA that can replicate autonomously in cells.

Plasmids offer many advantages as cloning vectors. They generally do not kill the cell and are relatively easy to purify to obtain the cloned DNA They can also be made relatively small, because they need very few plasmid-encoded functions for their replication. As a result, plasmids are particularly popular tools in some types of cloning applications. In fact, in one of the first DNA cloning experiments, a frog gene was cloned into plasmid pSC101. The replication origins and copy numbers for several E. coli plasmid vectors are given in Table 4.3.

TABLE 4.3 Replication origins of several *E. coli* plasmid vectors

Plasmid	ori	Copy number
pBR322	pMB1	15–20
pUC vectors	pMB1 mutant	100s
pET vectors	pMB1 mutant	100s
pBluescript	pMB1 mutant	100s
pACYC184	p15A	10–12
pSC101	pSC101	5

Desirable Features of Plasmid Cloning Vectors

Most naturally occurring plasmids are not convenient cloning vectors. However, plasmids can often be "engineered" to make a cloning vector. To be useful as a cloning vector, a plasmid should have at least some of the following properties

1. It should be small, so that it can be easily isolated and introduced into various bacteria.
2. It should have a relatively high copy number, so that it can be easily purified in sufficient quantities.
3. It should carry an easily selectable trait such as a gene conferring resistance to an antibiotic, which can be used to select cells that contain the plasmid.
4. It should have one or a few sites for restriction endonucleases, which cut DNA and allow the insertion of foreign DNAs. Also, these sites should ideally occur in selectable genes to facilitate the detection of plasmids that have foreign DNA inserts by a process called insertional inactivation.

Many plasmid cloning vectors have other special properties that aid in particular experiments. For example, some contain the sequences recognized by phage packaging systems (pac or cos sites), so that they can be packaged into phage heads (see chapter 7). Expression vectors can be used to make foreign proteins in bacteria Mobilizable plasmids have mobilization (mob) sites and so can be transferred by conjugation to other cells (see chapter 5). Some broad-host-range vectors have ori regions that allow them to replicate in many types of bacteria or even in organisms from different kingdoms Shuttle vectors contain more than one type of replication origin and so can replicate in unrelated organisms.

CLONING VECTOR pBR322

Figure 4.16 shows a map of pBR322, which embodies many of the desirable traits of a cloning vector. This plasmid is fairly small (only 4,360 bp) and has a relatively high copy number (~16 copies per cell), making it easy to

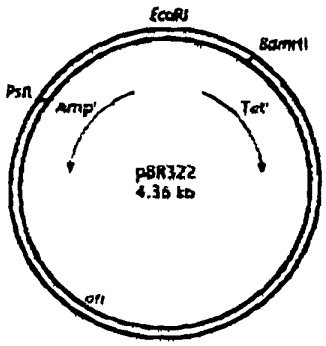


Figure 4.16 The plasmid cloning vector pBR322. Unique restriction sites are highlighted. Amp', ampicillin resistance; Tet', tetracycline resistance.

isolate The vector was constructed by removing all but the essential ori region from pMB1, a ColE1-like plasmid, and adding two resistance genes for the antibiotics tetracycline and ampicillin, which were taken from plasmid pSC101 and transposon Tn3, respectively. The plasmid also has several unique sites for restriction endonucleases, including BamHI, EcoRI, and PstI. These enzymes cut DNA at specific sites, allowing a piece of foreign DNA to be inserted into the plasmid and then studied.

Ordinarily in cloning experiments, most of the cloning vectors will not have picked up another piece of DNA and will remain unchanged. Some way is needed to detect cells that have received the vector with a piece of DNA inserted. Insertional inactivation offers a simple generic test for determining whether a cell contains a plasmid with a foreign DNA insert. In this technique, a gene is inactivated by having a piece of DNA inserted into it. To illustrate, Figure 4.17 shows a piece of foreign DNA inserted into the BamHI site in the tetracycline resistance (Tet') gene in pBR322 The piece of foreign DNA in the BamHI site will disrupt the Tetr gene and cause the plasmid to lose the ability to confer tetracycline resistance on a bacterium that carries it. The plasmid will still confer ampicillin resistance, however, since the Amp' gene remains intact. Therefore, cells containing a plasmid with a foreign DNA insert will be ampicillin resistant but tetracycline sensitive, which is easy to test on agar plates containing one or the other antibiotic.

Broad-Host-Range Cloning Vectors

Many of the common E. coli cloning vectors such as pBR322, the pUC plasmids, and the pET plasmids have been constructed with the pMB1 ori region and thus are very narrow in their host range. They will replicate only in E. coli and a few of its close relatives. However, some cloning applications require a plasmid cloning vector that will replicate in other gram-negative bacteria, and so cloning vectors have been derived from the broadhost-range plasmids RSF1010 and RP4, which will replicate in most gram-negative bacteria. In addition, to the broad-host-range ori region, these cloning 'Vectors sometimes contain a mob site called an oriT site, which can all w them to be transferred into other bacteria (see chapter 5). This latter trait is very useful, because other ways of introducing DNA have not been developed for many types of bacteria.

SHUTTLE VECTORS

Sometimes, a plasmid cloning vector from one organism must be transferred into another organism. If the two organisms are not related, the same plasmid ori region is not likely to function in both organisms. Such situations require the use of shuttle vectors, so named because they can be used to "shuttle" genes between the two organisms. A shuttle vector has two origins of replication, one that functions in each organism. Shuttle vectors; also must contain selectable genes that can be expressed in both organisms.

In most cases, one of the organisms in which the shuttle vector can replicate is E. coli. The generic tests can be performed in the other organism, but the plasmid can be purified and otherwise manipulated by the refined methods developed for E. coli.

Some shuttle vectors can replicate in gram-positive bacteria and E. coli, whereas others can be used in lower or even higher eukaryotes. For example, plasmid YEp13 (Figure 4.18) has the replication origin of the 2um circle, a plasmid found in the yeast Saccharomyces cerevisiae, so it will replicate in S. cerevisiae. It also has the pBR322 ori region and so will replicate in E. coli. In addition, the plasmid contains the yeast gene LEU2 as well as Ampr, which confers ampicillin resistance in E. coli. Similar shuttle vectors that can replicate in mammalian cells and E. coli have been constructed. Some of these plasmids have the replication origin of the animal virus simian virus 40 and the ColEl origin of replication

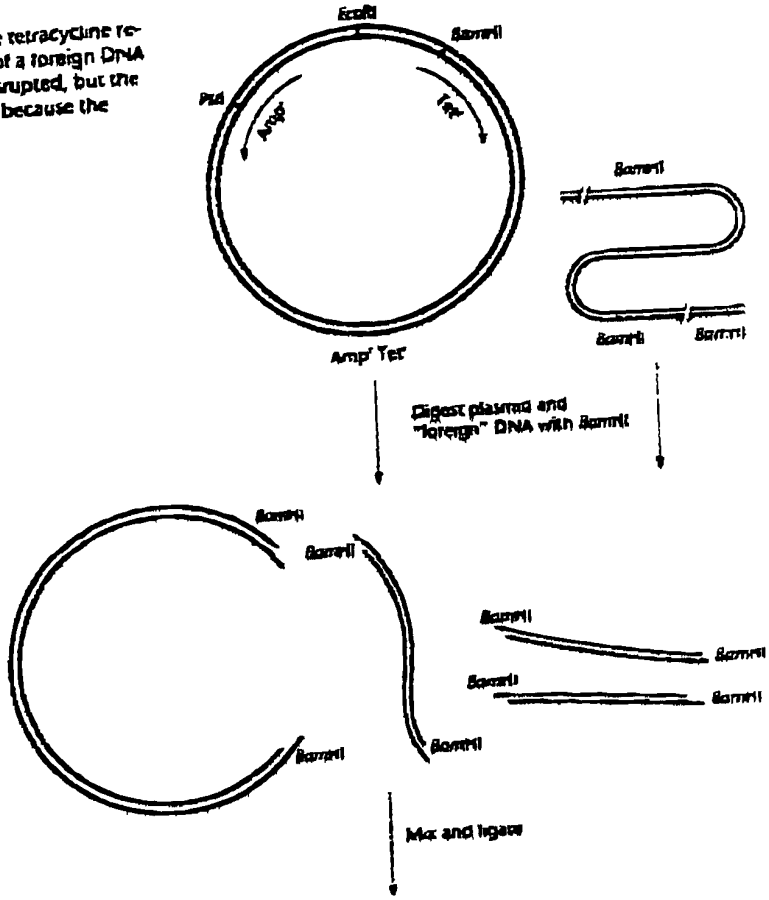
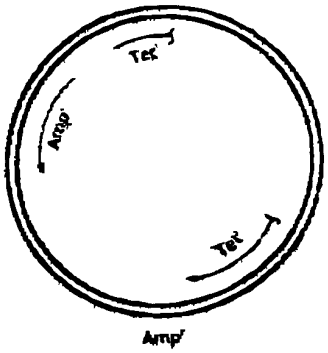


Figure 4.17 Insertional inactivation of the tetracycline resistance (Tet') gene of pBR322 by insertion of a foreign DNA into the *Bam*HI site. The Tet' gene will be disrupted, but the plasmid will still confer ampicillin resistance because the Amp' gene will still be active.



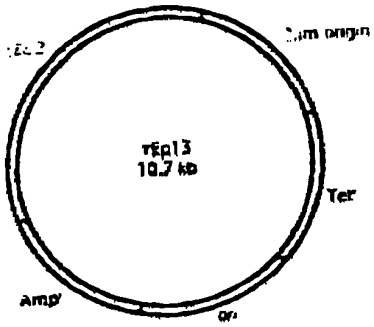


Figure 4.18 The shuttle plasmid YEp13. The plasmid contains origins of replication that will function in *S. cerevisiae* and *E. coli*. It also contains genes that can be selected in *S. cerevisiae* and *E. coli*.

COSMIDS

As mentioned, L packages DNA into its head by recognizing cos sites in concatemeric DNA; therefore, any DNA containing a cos sequence will be packaged into phage heads. In particular, plasmids containing cos sites can be packaged into X phage heads. Such plasmid cloning vectors, called cosmids, also offer many advantages for generic engineering, including in vitro packaging. In this procedure, plasmid DNA is mixed with extracts of L-infected cells containing heads and tails of the phage. The DNA will be taken up by the heads, and because L particles will self-assemble in the test tube, the tails will be attached to the heads to make infectious L particles, which can then be introduced into cells. Any L

cloning vector will serve in this method, and infection is a more efficient way of introducing DNA into bacteria than is transfection or transformation.

Another major advantage of cosmids is that the size of the cloned DNA is limited by the size of the phage head. If the piece of DNA cloned into a cosmid is too large, the cos sites will be too far apart and the DNA will be too long to fit into a phage head. However, if the cloned DNA is too small, the cos sites will be too close to each other and the phage heads will have too little DNA and be unstable. Therefore, the use of cosmids ensures that the pieces of DNA cloned into a vector will all be approximately the same size, which is sometimes important for making libraries (see chapter 15).

Appendices

Box 4.3: Plasmid Addicti n

Even with their partitioning functions, plasmids are lost from multiplying cells. In a kind of ironic revenge, some plasmids encode proteins that kill cells cured of their type. Such functions have been described for the F plasmid, the R1 plasmid, and the P1 prophage, which replicates as a plasmid. These plasmids encode a toxic protein that will kill the cell if it is expressed. The toxic protein kills the cell by various mechanisms, depending on the source. For example, the toxic protein of the F plasmid, Ccd, kills the cells by altering DNA gyrase, so it causes double-stranded breaks in the DNA. The killer protein, Hok, of the plasmid R1 destroys the cellular membrane potential, causing loss of cellular energy. The mechanism of killing by the P1-encoded killer protein, Doc, is not known, but it does not seem to work by either of the above mechanisms.

Why does the toxic protein kill cells only immediately after they have been cured of the plasmid? When cells contain the plasmid, other proteins or RNAs of the addiction system act as an antidote and either prevent the synthesis of the toxic protein or bind to it and prevent its activity. These other gene products are much more unstable than the toxic protein, however, so that they will be more rapidly inactivated. If a cell is cured of the plasmid, neither the toxic protein nor the antidote will be synthesized, but since the antidote is more unstable, it will not be long before the cured cells have only the toxic protein and are killed. These systems make the cell addicted to the plasmid once it has been acquired, and they prevent cured cells from accumulating.

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Box 4.2: Plasmids and Bacterial Pathogenesis

Plasmids often carry virulence genes required for bacterial pathogenicity. For example, to be pathogenic, strains of the genus *Shigella* must carry a large plasmid longer than 200 kb that contains genes for cell invasion and cell adhesion. Interestingly, the genes for regulating the virulence genes on the plasmids are in the chromosome rather than in the plasmid. The genes for the Shiga toxin are also in the chromosome. The Shiga toxin is related structurally to the diphtheria toxin but has an N-glycosylase activity that removes a specific adenine base in the 28S rRNA, thus blocking translation. This distribution of virulence genes between the plasmid and the chromosome in strains of *Shigella* demonstrates the close relationship between plasmids and their hosts.

Most strains of *Salmonella* also require a large plasmid for their pathogenicity. However, the precise virulence genes that are carried on the plasmid are not known, and *Salmonella typhi* does not require a plasmid to cause typhoid fever.

One of the clearest examples of plasmid virulence genes is in the genus *Yersinia*. The three species of *Yersinia*, *Y. enterocolitica*, *Y pseudotuberculosis*, and *Y pestis*, all cause disease, ranging in severity from mild enteritis in the case of *Y. enterocolitica* and *Y pseudotuberculosis* to bubonic plague in the case of *Y. pestis*. To be pathogenic, all three species must harbor closely related plasmids that are about 70 kb long.

These plasmids encode outer membrane proteins called Yops, which may help the bacteria avoid host phagocytosis. The Yops are synthesized only under conditions of limiting calcium ions and high concentrations of sodium ions – conditions that may mimic the environment inside eukaryotic cells. The "plague bacillus," *Y. pestis*, must also carry two other smaller plasmids to cause disease. One of these encodes a toxin and an antiphagocytic protein similar to Yops, and the other encodes a protease that increases invasiveness. *Y. pestis* also has an iron-scavenging system that allows it to extract iron directly from hemin. The ion-scavenging system is encoded by a large, normally nonessential, chromosomal element that might be an integrated plasmid or prophage. Why so many genes required for pathogenesis are carried on plasmids and other DNA elements is a subject of speculation.

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